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REVIEW

Mechanisms of immune checkpoint inhibitor-mediated liver injury

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Abstract The immune checkpoints, cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death protein-1/ligand-1 (PD-1/PD-L1) are vital contributors to immune regulation and tolerance. Recently immune checkpoint inhibitors (ICIs) have revolutionized cancer therapy; however, they come with the cost of immune related adverse events involving multiple organs such as the liver. Due to its constant exposure to foreign antigens, the liver has evolved a high capacity for immune tolerance, therefore, blockade of the immune checkpoints can result in aberrant immune activation affecting the liver in up to 20% of patients depending on the agent(s) used and underlying factors. This type of hepatotoxicity is termed immune mediated liver injury from checkpoint inhibitors (ILICI) and is more common when CTLA4 and PD-1/PD-L1 are used in combination. The underlying mechanisms of this unique type of hepatotoxicity are not fully understood; however, the contribution of $CD8^+$ cytotoxic T lymphocytes, various $CD4^+$ T cells populations, cytokines, and the secondary activation of the innate immune system leading to liver injury have all been suggested. This review summarizes our current understanding of the underlying mechanisms of liver injury in immunotherapy using animal models of ILICI and available patient data from clinical studies.

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1. Introduction

The immune checkpoint refers to regulatory steps within the immune system that prevent auto-reactivity of T lymphocytes and promote self-tolerance. The cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) and programmed cell death protein-1 (PD-1) and its ligand (PD-L1) are the main negative regulators of T cell immune function[1]. Immune checkpoint inhibitors are designed to target one of these molecules and result in increased T cell activation against tumor cells, which upregulate these pathways to evade immunity (Fig. 1). While these treatments, alone and in combination, have been groundbreaking in solid tumor cancer therapy, they come at the cost of several immune-related adverse events (irAE) due to disruption of self-tolerance[1]. One such complication is immune-mediated liver injury from checkpoint inhibitors (ILICI)[1]. ILICI, its clinical presentation, and management have been reviewed detail[1]. In this review, we aim to discuss the underlying mechanisms of this form of immune-mediated hepatotoxicity in animal models and humans.

1.1. Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4)

CTLA-4 transmembrane glycoprotein is an inhibitory molecule that attenuates T cell activation. The co-stimulatory receptor CD28 and its ligands B7-1 (CD80) and B7-2 (CD86) represent a model integrating both stimulatory and inhibitory interactions involving T cells[2]. The discovery of CTLA-4, which like CD28 binds to B7-1 and B7-2, has evolved our understanding of this complex regulatory system[2]. CD28 is expressed constitutively on the cell surface of naive $CD4^+$ and $CD8^+$ T cells. It provides an indispensable co-stimulatory signal for T cell growth and endurance upon ligation by B7-1 and B7-2 on antigen-presenting cells (APCs)[3]. CTLA-4 is expressed following T cell activation and its upregulation results in CD28 downregulation by endocytosis[3]. The balance between stimulatory and inhibitory signals determines the T cell response[3]. By restricting contact between T cells and APCs and reducing the collection of T cell receptor (TCR) molecules in the immunological synapses, CTLA-4 can prevent TCR signaling[3].

CTLA-4 can be expressed within the tumor microenvironment on infiltrating regulatory T cells (Tregs) or exhausted T cells, and on tumor cells[4]. The inhibitory effects of Tregs on cytotoxic T lymphocytes (CTLs) is carried out in part by the CTLA-4 signaling pathway, thus leading to the suppression of effector T cells[4,5]. Inhibition of the CTLA-4 signaling pathway in tumor-specific cytotoxic T cells allows them to escape exhaustion and to enter a proliferative effector phase. These activated effector T cells can infiltrate the tumor microenvironment and secrete cytokines, such as interferon-γ (IFN-γ), tumor necrosis factor (TNF), and interleukin-2 (IL-2), etc., creating an immunogenic environment[6].

1.2. Programmed cell death protein-1/ligand-1 (PD-1/PD-L1)

PD-1, CD279 is a co-inhibitory receptor that is expressed on the surface of antigen-stimulated T cells. PD-1 has interactions with two ligands: PD-L1 (CD274) and PD-L2 (CD273)[7,8]. PD-1 is expressed by hematopoietic cells, but its ligand, PD-L1, has been detected in the liver especially with chronic inflammatory liver conditions such as viral and autoimmune hepatitis[9]. The expression of PD-L1 on hepatic stellate cells (HSCs), liver sinusoidal epithelial cells (LSECs), Kupffer cells (KCs), and vascular endothelial cells correlates directly with the degree of liver inflammation[10–12]. PD-L1 expression on hepatocytes has also been noted but remains more controversial[9]. The binding of PD-1 to PD-L1 inhibits the production of IFN-γ, TNF and IL-2, prevents the proliferation of T cells, and also reduces T cell survival[13]. PD-1/PD-L1 interactions have been shown to diminish the

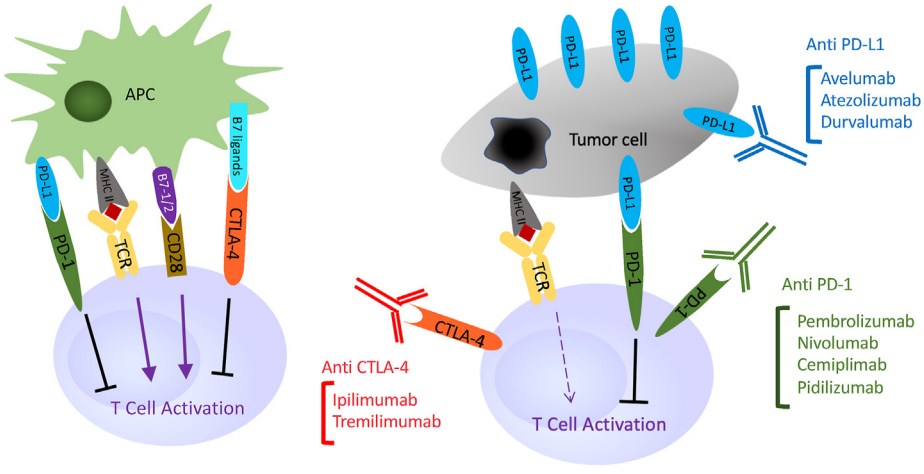

Figure 1 Immune checkpoint inhibitors, site of action. Antigen presentation by major histocompatibility complex (MHC) class II molecules on antigen presenting cells (APCs) leads to antigen recognition by the T cell receptor (TCR) and T cell activation. B7-1/2 binding to CD28 surface receptor further modulates T cell activation signals. CTLA-4 located on the T cell surface competes for CD28 receptor binding to block T cell activation. CTLA-4 inhibitors, ipilimumab, and tremelimumab interfere with this interplay and promote T-cell activation. PD-1/PD-L1 interactions have been shown to diminish the activity of mature T cells after T cell receptor signaling has been initiated. PD-1 and PD-L1 inhibitors such as pembrolizumab, nivolumab, cemiplimab, pidilizumab, avelumab, atezolizumab and durvalumab function by blocking the PD-1/PD-L1 interaction thereby promoting T-cell activation and survival. APC, antigen presenting cells; CTLA-4, The cytotoxic T-lymphocyte-associated antigen-4; MHC, major histocompatibility complex; PD-1, programmed cell death protein-1; PD-L1, programmed cell death protein ligand 1; TCR, T cell receptor.

Table 1 Cancers and the generic and brand name immune-checkpoint inhibitors (ICIs) commonly utilized for their treatment sorted by target.

ICI generic name	ICI brand name	ICI target	Malignancies
PD-1 and PD-L1 inhibitor			
Pembrolizumab	Keytruda[22]	PD-1	Melanoma[22], MCC[22], NSCLC[22], CHL[22], PMBCL[22,27], urothelial carcinoma[22], HCC[22,27], gastric carcinoma[22], HNSCC[22], CC[22], MSI-H solid tumors[22], dMMR solid tumors[22]
Nivolumab	Opdivo[22]	PD-1	Melanoma[22], NSCLC[22], ovarian carcinoma[28], CHL[22], urothelial carcinoma[22], RCC[22], HCC[22], HNSCC[22], MSI-H CRC[22], dMMR CRC[22]
Cemiplimab	Libtayo[22]	PD-1	Metastatic or locally advanced CSCC[22]
Pidilizumab	–	PD-1	DLBCL[29], B cell lymphoma[28], follicular lymphoma[28]
Avelumab	Bavencio[22]	PD-L1	MCC[22], urothelial carcinoma[22]
Atezolizumab	Tecentriq[22]	PD-L1	NSCLC[22,27], urothelial carcinoma[22,27]
Durvalumab	Imfinzi[22]	PD-L1	NSCLC[22], urothelial carcinoma[22], HCC[30]
CTLA-4 inhibitor			
Ipilimumab	Yervoy[22]	CTLA-4	Melanoma[22], RCC[22], MSI-H CRC[22], dMMR CRC[1]
Tremelimumab (formerly ticilimumab)	–	CTLA-4	Melanoma[28], NSCLC[22], HCC[30]

CC, cervical carcinoma; CHL, classical Hodgkin lymphoma; CRC, colorectal carcinoma; CSCC, cutaneous squamous cell carcinoma; DLBCL, diffuse large B-cell lymphoma; dMMR, DNA mismatch repair deficiency; HCC, hepatocellular carcinoma; HNSCC, head and neck squamous cell carcinoma; MCC, Merkel cell carcinoma; MSI-H, microsatellite instability-high; NSCLC, non-small cell lung carcinoma; PMBCL, primary mediastinal large B-cell lymphoma; RCC, renal cell carcinoma; SCLC, small cell lung carcinoma.

activity of mature T cells after T cell receptor (TCR) signaling has been initiated. In contrast, PD-1/PD-L1 interactions do not seem to affect naive B or T cells. When PD-1 is upregulated, previously-activated T cells become exhausted and are thus rendered less efficient[13,14].

Cancer and chronic infections can lead to chronic T cell activation through continuous antigen presentation by APCs. Upon identification of an antigen, T cells promptly express PD-1[15]. The constant activation of T cells produces a state of exhaustion and subsequent T cell malfunction, resulting in inadequate control of infections and tumors[13,14]. Increased expression of PD-1 is a defining feature of T cell exhaustion[16]. PD-L1 expression on tumor cells is not constant and can be swiftly reduced[17]. The upregulation of PD-L1 on cancerous tissue blunts T cell responses, seemingly aiding cancer cells to adjust and survive in a drastically pro-inflammatory cancer microenvironment by evading the immune system.

2. Immune-checkpoint inhibitors (ICIs)

Immune-checkpoint inhibitors (ICIs) have revolutionized anti-tumor therapy and have become a first-line therapy for the treatment of various cancers. These monoclonal antibodies function to restore the ability of the immune system to detect tumors by manipulating the interaction between tumor cells and immune checkpoints to illicit an appropriate antitumor immune response. Tumor cells frequently manipulate these two checkpoints to evade immune surveillance and promote their survival. As a result, developing antibodies against these checkpoints has been pivotal in creating new cancer therapies to promote tumor cell death by causing hyperactivation of CTLs and downregulating tolerogenic Tregs[18]. Ipilimumab, a monoclonal anti CTLA-4 antibody was the first ICI approved in the US in 2011 for melanoma[19]. In 2008 Berger et al.[20] demonstrated that PD-L1 antibodies are efficacious in treating hematologic malignancies. In 2010, PD-1-inhibiting drugs were proven effective against various cancers such as colorectal cancer, melanoma, renal cell carcinoma, non-small cell lung cancer, and prostate cancer[21]. At the time, checkpoint inhibitors had a satisfactory toxicity profile, and were shown to have high efficacy in reducing cancer burden[21]. Other antibodies were subsequently developed to target the CTLA-4 and PD-1 checkpoints (Table 1)[22–25]. Most of these immunotherapies have been used successfully in the clinic, with the exception of Tremelimumab, which failed phase III clinical trials in melanoma patients[26].

3. Immunotolerance in the liver

The liver encounters a great quantity and variety of antigens every day to fulfill its physiological functions. As a result, the liver has evolved several mechanisms to dampen immune reactions and promote local immune tolerance[31–33]. Resident liver cells such as LSECs and KCs function as scavenger cells and APCs affecting the immune response by modulating leukocyte recruitment[31,32]. Dendritic cells (DC), which are also agents of liver immune tolerance, are recruited by KC *via* the liver lymphatics and remain in the liver to become resident DCs[32]. Immune cell attraction to the liver is tightly regulated and is antigen-specific to prevent aberrant nonspecific autoimmune responses. The liver constitutively expresses Toll-like receptors (TLRs) due to its constant exposure to lipopolysaccharides (LPS) and other pathogen associated molecular patterns (PAMPs)[34]. In order to prevent the development of inflammation, the liver has evolved a hyporesponsive state towards PAMPs, termed "endotoxin tolerance", which is achieved by immune regulatory cytokines such as IL-10, tumor growth factor-β (TGF-β), and negative regulators of TLR signaling[32,35]. Liver resident DCs express low levels of major histocompatibility complex-II (MHC-II) and costimulatory molecules (such as CD80/CD86) compared to their lymphoid resident counterparts and secrete less IL-12 and preferentially secrete immune-tolerogenic cytokines such as IL-10 and IL-27[36,37] (Fig. 2). This synergizes with silencing of bioactive IL-12 activity leading to profound T cell inhibition[36]. DCs also promote Treg differentiation by secreting IL-10 among other immune regulatory functions[36]. In addition to

liver resident DCs, LSECs promote an immunotolerant liver microenvironment by activating naïve $CD4^+$ T cells, secreting IL-10, promoting a Th0 phenotype over the more proinflammatory Th1 phenotype[38]. Like DCs, LSECs function as scavengers and APCs, and even employ molecular mechanisms of antigen cross-presentation to $CD8^+$ T cells. Interestingly, the outcome of this is T cell tolerance rather than immune reactivity[39,40]. Indeed, LSECs suppress Th-mediated immunity and induce the development of forkhead box P3 (FOXP3) Tregs[41] (Fig. 2).

The KC population which are liver resident macrophages, also provide important immune tolerance functions and are known to produce IL-10 in response to endotoxin exposure that is autoregulated at the transcriptional level[42]. KCs stimulate the suppressive activity of Tregs and induce IL-10 expression by these cells, which is crucial for the induction of tolerance to hepatocyte-expressed antigens[32,43]. KCs are required for intrahepatic clonal deletion of activated CTLs by apoptosis[44]. While KCs support tolerance induction under basal conditions, their interaction with T cells and NKT cells in the context of infection and injury can mediate the development of an antimicrobial and inflammatory phenotype to fight pathogens[32]. Hepatic stellate cells can also contribute to the tolerogenic liver microenvironment by serving as APCs, expanding the inhibitory Tregs, increasing T-cell apoptosis *via* PD-L1 engagement, and inhibiting CTLs by a CD54 dependent mechanism (Fig. 2)[45–48].

Hepatocytes also participate in the promotion of immunotolerance. Although they are not classical APCs, hepatocytes can present antigens and prime naïve $CD8^+$ T cells owing to their large size and due to the sinusoidal fenestrations resulting in close contact with lymphocytes and other circulating cells. These T cells may undergo initial expansion after contact but due to a lack of sufficient co-stimulation they subsequently undergo BCL2 interacting mediator (BIM)-mediated apoptosis and clonal deletion resulting ultimately in immune tolerance[49,50]. The interaction of hepatocytes with NKT cells leads to the generation of IL-10 expressing cells with regulatory function[51,52].

An important mechanism of liver immunotolerance is the expression of PD-L1 and PD-L2 on non-parenchymal cells in the liver including hepatic stellate cells (HSC), Kupffer cells, LSECs, intrahepatic white blood cells. Although baseline expression of PD-L1 on liver parenchymal cells is controversial, induction of PD-L1 on hepatocytes in inflammatory diseases such as autoimmune and viral hepatitis has also been reported[53,54]. Increased PD-L1 expression on hepatocytes seems to be stimulated by interferons[53]. It is possible that PD-L1 expression is upregulated in hepatocytes in these disease conditions as a compensatory mechanism to promote immune tolerance as PD-L1 levels were noted to be higher in AIH patients who responded to medical therapy[53]. PD-L1 expression on LSECs is critical for induction of $CD8^+$ T cell apoptosis, as PD-L1 deficient LSECs were incapable

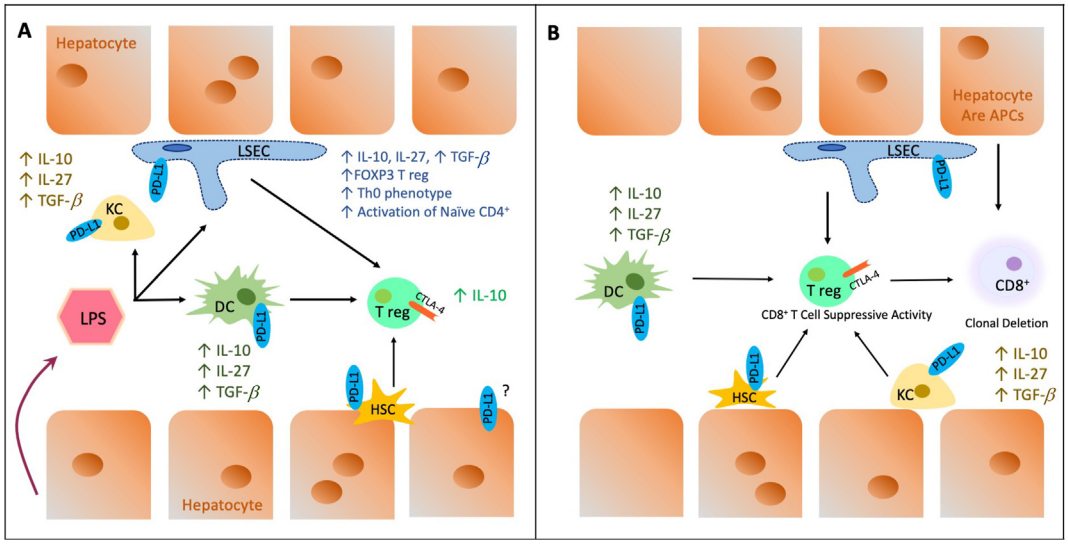

Figure 2 Immune tolerance in the liver. Several mechanisms and types of cells contribute to the tolerogenic environment in the liver. (A) Constant low dose exposure to pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) from the intestine prime the liver cell population and promote an immunotolerant microenvironment. LPS promotes a state of immune tolerance by affecting the secretion on IL-10, IL-27 and TGF-β by multiple cells including dendritic cell (DCs), liver sinusoidal endothelial cells (LSECs), and Kupffer cells (KC). LSECs secrete anti-inflammatory cytokines and promote Th0 phenotype and FOXP3 Tregs. They activate naïve $CD4^+$ T cells which also secrete IL-10. Non-parenchymal liver cells have been shown to express PD-L1. Hepatocytes also participate in immune tolerance, although the level of PD-L1 expression on healthy and unstimulated liver parenchymal cells is controversial. (B) Another mechanism of immune tolerance induction is suppression of $CD8^+$ CTLs. Hepatocytes can act as APCs and activate naïve $CD8^+$ T cells that ultimately undergo apoptosis and clonal deletion due to lack of sufficient co-stimulation. PD-L1 expression on liver non-parenchymal cells is critical for induction of $CD8^+$ T cell apoptosis. KCs, LSECs, and hepatic stellate cells (HSCs) increase $CD4^+$ regulatory T cells (Tregs) suppressive activity and cause clonal deletion of cytotoxic T lymphocytes (CTLs) by apoptosis. CTLA-4 expression on $CD4^+$ Tregs contributes to maintenance of liver immune tolerance by downregulating $CD8^+$ CTLs. APC, antigen presenting cell; CTL, cytotoxic T lymphocyte; CTLA-4, cytotoxic T lymphocyte associated antigen 4; DC, dendritic cell; FOXP3, forkhead box P3; HSC, hepatic stellate cells; IL-10, interleukin 10; IL-27, interleukin 27; KC, Kupffer cell; LPS, lipopolysaccharide; LSEC, liver sinusoidal endothelial cell; PAMPs, pathogen associated molecular patterns; PD-L1, programmed cell death protein ligand 1; Treg, regulatory T cell; TGF-β, transforming growth factor beta.

of inducing T cell tolerance[12]. The expression of PD-L1 on these cells together with the expression of CTLA-4 on $CD4^+$ Tregs helps protect the liver from autoimmune responses to antigens by downregulating effector T cells[55], either by induction of T cell apoptosis or causing T cell dysfunction or a failure to develop cytotoxic effector function (Fig. 2)[10,12,51,53,56].

Given the importance of these checkpoint proteins in the induction of immune tolerance during homeostatic conditions, it is not surprising that inhibiting these pathways affects the liver's capacity for immune regulation and induction of tolerance. Germline deletion of CTLA-4 ($CTLA\text{-}4^{-/-}$) results in a lethal lymphoproliferative disorder and multi-organ accumulation of self-reactive T lymphocytes in the heart, pancreas, and the liver[57,58]. Furthermore, single nucleotide polymorphisms and mutations in the *CTLA-4* gene have been implicated in multiple autoimmune diseases including autoimmune hepatitis and primary biliary cholangitis (PBC)[59,60]. This clear clinical associations with autoimmune disorders highlight the importance of these pathways and mechanisms of immune tolerance in liver homeostasis.

4. Immune-mediated liver injury caused by checkpoint inhibitors (ILICI)

The blockade of CTLA-4, PD-1, or PD-L1 triggers the immune activities of $CD8^+$ CTLs that target a broad spectrum of self-antigens[61]. This activation of autoreactive T cells is complex and causes an extensive pattern of autoimmune adverse effects. Not all patients who receive ICIs develop toxicity and while not yet fully understood, certain risk factors have been associated with more side effects. Factors that influence irAEs include: the drug[62], mono *vs*. combination therapy[62], sex[62], age[62], genetic predisposition[62], co-morbidities[62], the effect of the diet and potentially gut microbiota[62], and organ-specific factors[62]. These adverse effects can involve single or multiple organs and have been reported in the gastrointestinal tract, liver, endocrine system, hypophysis, skin, adrenals, kidneys, lungs, and nervous system. Ophthalmological, rheumatological and hematological side effects have been reported as well[63].

Immune-mediated liver injury caused by checkpoint inhibitors (ILICI) is a recently described form on immune-mediated drug-induced liver injury (DILI)[1,64]. ILICI differs from direct and idiosyncratic DILI (IDILI) based on the underlying mechanism, clinical signs, symptoms, and management[1,64]. The incidence of ILICI varies depending on multiple risk factors such as type and dosage of ICI, monotherapy, or combination therapy (with other ICIs or small molecule inhibitors), genetic predisposition, pharmacotherapy, exposure to acetaminophen, and statins[65]. Younger age, male sex, and pre-existing co-morbidities such as autoimmune diseases have been shown to influence the incidence, time of onset and grade of hepatotoxicity[65–68]. Similar to IDILI, ILICI is a diagnosis of exclusion[69,70]. The grade of hepatotoxicity from ICIs is generally graded by the Common Terminology Criteria for Adverse Events (CTCAE) v5.0[71]. Liver injury ranges from grade 1 with minimal liver enzyme elevations to severe liver injury requiring IV steroids. In patients with abnormal baseline values such as Gilbert's diseases, fold abnormality should be considered relative to baseline values[1].

The development of ILICI above grade 2 requires stopping therapy and any level of toxicity beyond grade 3 or 4 adverse events indicates the need for permanent discontinuation of ICIs, initiation of corticosteroids, and close monitoring of the liver panel. If no decrease in liver enzymes is observed within 3−5 days of ICI discontinuation, the use of other immunosuppressive therapies may be considered[72].

Although ICI treatment has become commonplace for various types of cancers, they have only recently been studied in hepatocellular carcinoma (HCC)[73–75]. Intrahepatic lymphocytes in the setting of chronic inflammation of the liver have been shown to overexpress PD-1, while KC, LSEC and even hepatocytes have been shown to upregulate PD-L1[9,11,76]. This upregulation of immune checkpoint markers in the setting of chronic liver disease, has made ICI therapy for HCC an attractive option. However, until recently, chronic viral infections [hepatitis B virus (HBV) and hepatitis C virus (HCV)] were exclusion criteria for ICI therapy. Recent data suggest that immunotherapy in the setting of antiviral treatment (optimally continued for at least 6 months after ICIs), does not increase viral reactivation, making immunotherapy a possibility in this setting[23,76–79]. Viral load has even been shown to decrease in several HCV RNA-positive patients after ICI therapy; however, the mechanism is unclear[25,77]. As ICIs are being increasingly used in patients with underlying liver disease and HCC, hepatotoxicity will become an even more important clinical issue, as ILICI and elevated aspartate aminotransferase (AST)/alanine aminotransferase (ALT) levels have been shown to comprise the majority of significant irAEs from ICI therapy in the treatment of HCC[76,80,81].

4.1. Mechanisms of immune checkpoint inhibitor-mediated toxicity

While the activation of CTLs is broadly known to contribute to all irAEs from ICIs, the underlying signaling pathways leading to hepatocellular injury and ILICI have not been fully elucidated. Given the main presentation of liver injury in this form of hepatotoxicity is elevated AST and ALT (accompanied in severe cases by an elevated bilirubin), injury to hepatocytes leading to damage and cell death is likely a major contributor. As discussed earlier, the primary proposed mechanism of all irAEs in this setting is based on the alteration of self-tolerance and T-cell mediated immune system activation. How hepatocytes are targeted, whether cell death is the result of direct engagement by CTLs or indirectly due to an effect on T helper population and Tregs, a proinflammatory cytokine milieu and activation of innate immunity is not fully understood.

4.1.1. ICIs and cytotoxic T lymphocytes

One of the mechanisms behind irAEs from immunotherapy is attributed to the process of epitope spreading (ES)[82]. ES is the diversification of the immune response from the original, targeted, epitope-specific response, to a more indiscriminate immune reaction to other proteins and self-antigens[82]. In this process, the lysis of tumor cells from such immune-mediated therapy causes the release of numerous proteins and host antigens (possibly newly generated neoantigens) which are then taken up by host APCs. Subsequently, a larger secondary pool of T cells with a greater diversity of repertoire than the original, are recruited and activated against these self-antigens, mediating injury[82,83]. It is thought that anti-CTLA-4 therapy results in more far-reaching and nonspecific effects on ES than anti-PD-1 therapy, as CTLA-4 plays an instrumental role in the activation of T cells[62]. The anti-CTLA-4 antibody, ipilimumab, induces a more significant diversification and clonal expansion of T cells in patients with irAEs, which may cause these activated T cells to target host tissue with a greater affinity than for tumor cells[84,85]. Additionally,

previous findings of similar T cells infiltrating both tumors and tissues in irAEs with an overlap of T cell antigens between the two, further support the notion that this off target effect plays a crucial role in initiating irAEs in susceptible patients[62]. PD-L1 is widely expressed in peripheral tissues to prevent $CD8^+$ T cells from attacking host tissue. Therefore, the blockade of the PD-1 pathway through the utilization of anti-PD-1/PD-L1 immunotherapies overcomes immune tolerance by stimulating the proliferation of $CD8^+$ cells[86,87] and altering their transcriptional profile to induce the upregulation of proliferative and cytotoxic genes such as IFN-γ, granzyme and granulysin[88,89]. These altered lymphocytes then theoretically directly target healthy host tissue, resulting in irAEs[62]. In a mouse model harboring the humanized CTLA-4 gene, using ipilimumab and anti PD-1, the degree of irAE corresponded to systemic T cell activation and resulted in reduced ratios of Treg to CTLs[90]. By studying humanized mice, either homozygous or heterozygous for the human allele, the results suggested therapeutic anti-tumor effects of anti-CTLA-4 antibody required only monoallelic engagement of *CTLA-4* gene but irAEs required bi-allelic engagement[90]. Bi-allelic blockade or deletion is key in preventing the conversion of autoreactive T cells to Treg, thus driving autoimmunity[90].

4.1.2. *The effect of ICIs on Thelper cells the Treg population*

The administration of ipilimumab has been shown to reduce the amount of Tregs present in tumor cells while increasing the number of effector T cells. Preclinical trials have further found a negative correlation between the number of Tregs and the incidence rates of irAEs[91–93]. In addition to cytolytic destruction of effector T cells, Tregs function in large part by secreting anti-inflammatory cytokines (such as IL-10, IL-35, TGF-β, etc.), and modulating the immune response by affecting DCs, monocytes and macrophages[94]. Therefore they may play a key role in the adaptive-innate immune interactions leading to irAEs. In addition to effects on Tregs, ICIs affect the recruitment of a great variety of T helper cell subpopulations, including Th1, Th2, Th17, T follicular helper cells[95]. Anti-CTLA-4 therapy has been shown to induce the expansion of Th1 cells thereby increasing the levels of proinflammatory cytokines (IL-2, IFN-γ, TNF) production, which can go on to activate CTLs, as well as innate immune cells such as macrophages and natural killer cells[62]. This indirect effect on innate immune cells *via* altering Th-mediated cytokine production may be particularly important in ILICI and hepatotoxicity where monocytes have been implicated in the pathogenesis of injury[96]. Another example of immune-related toxicity from the indirect effect of ICIs on innate immunity was the observed correlation of neutrophil activation markers such as CD177 and carcinoembryonic antigen-related cell adhesion molecule (CEACAM1) in the sera of a subset of patients who went on to develop ipilimumab-induced colitis and GI toxicity[97]. In a prospective study of patients with irAEs, a strong association between developing irAEs and C−X−C motif chemokine ligand (CXCL) 9, 10, 11 and 13, which regulate the differentiation of naïve T cells into Th1 cells was noted[98]. The role of the Th population and inflammatory cytokines is important and provides potential clues in the innate and adaptive immune crosstalk in irAEs. Anti-CTLA-4 antibodies also bind to CTLA-4 expressed on Tregs, causing depletion of Tregs through antibody-dependent cell-mediated cytotoxicity[62]. Loss of Tregs results in dysregulation of immune responses, which is also potentially involved in the pathogenesis of irAEs[62]. The complex development of $CD4^+$ T cell subtypes as a result of ICIs and their contribution to the development of irAEs has been reviewed in detail elsewhere[62].

4.1.3. *ICI effects on B cells*

Evaluating B cell genomic profiles of patients receiving dual checkpoint inhibition therapy for melanoma revealed an early decline in total circulating B cells; however, further analysis revealed an elevation in a $CD21^{lo}$ subtype known to be anergic and an exhausted B cell subtype[99]. The increase in this subtype of B cells has also been detected in CTLA-4 deficient patients[100]. A nonsignificant association has been reported between autoantibody development and the probability of irAEs in patients undergoing ipilimumab immunotherapy for melanoma[101]. These data may suggest a role for B cells in checkpoint inhibitor efficacy and toxicity; however, further research is needed to elucidate the underlying mechanisms.

4.1.4. *Cytokines and the contribution of innate immunity and the adaptive-innate cross talk*

The role of circulating cytokines as biomarkers and predictors of irAEs has been studied[102]. Cytokines can induce the expression of PD-1 and other checkpoint molecules such as T cell immunoglobulin domain and mucin domain-3 (TIM-3)[103]. Baseline elevated IL-17 levels has been shown to predict irAEs such as enterocolitis. In a study using dual blockade of PD-L1 and CTLA-4 in melanoma patients, the expression of eleven cytokines was integrated into a single cytokine toxicity score and was predictive of the severity of adverse events resulting from ICI therapy[102]. Inflammatory cytokines such as IL1a, IL2, IFNα were increased at baseline and early on during ICI therapy. While elevated cytokines predicted irAEs they did not in all cases correlate with response to immunotherapy[102]. The fact that baseline cytokine levels were elevated in patients who developed irAEs (including five with hepatitis), points to a possible mechanistic role for cytokines in modulating an auto-immune response. Taken together with recent data implicating monocytes in the pathogenesis of ILICI, a more thorough investigation of the relation between T cell responses, elevated cytokine levels and the role of innate immune cell activation and recruitment in these cases is warranted.

An interesting possible role for TNF in mediating ILICI has been suggested. Clinically, anti-TNFs have been used to treat ICI-mediated enterocolitis[104]. In a study conducted by Perez-Ruiz et al.[105] on a humanized mouse colon cancer model, prophylactic TNF blockade with etanercept, reduced colitis as well as hepatitis from combination therapy of anti-CTLA-4 and anti-PD-1 immunotherapy. Importantly, concomitant etanercept administration did not diminish the therapeutic effect of ICIs[105]. A recent study by Badran et al.[106] further lends support to the significant role of TNF in mediating adverse events, specifically in ICI-mediated colitis. In this small case series, patients who developed ILICI were treated with infliximab to prevent prolonged steroid use[106]. Anti-TNF therapy was safe and effective, preventing gastrointestinal irAEs, while cancer treatment efficacy was not affected[106]. Whether TNF serves to activate innate immunity or directly causes hepatotoxicity *via* death receptor mediated signaling, causing inflammation and cell death is not known (Table 2).

Affolter and colleagues used an animal model of ILICI to study the underlying mechanisms leading to liver injury[108]. They attempted the simultaneous blockade of CTLA-4, PD-1 and indoleamine 2,3-dioxygenase1 (IDO1). Global knockout (KO) of

PD-1 in mice did not result in aberrant liver enzymes, however, small foci of immune cell infiltration were observed[108]. Treatment of PD-1 KO mice with CTLA-4 and IDO-1 inhibitors led to elevated glutamate dehydrogenase and the infiltration of both $CD4^+$ and $CD8^+$ T cells associated with areas of hepatocyte necrosis[108]. These areas of hepatocyte dropout were surrounded by mononuclear cells[108]. While this study did not find a role for innate immune cells, it noted a decrease in the number of macrophages[108]. Transcriptomics and pathway analysis suggested the activation of both apoptotic and necrotic pathways in the liver, as well as oxidative stress signaling, Fcγ receptor-mediated phagocytosis, C−C chemokine receptor type 5 (CCR5) signaling, as contributors to liver inflammation in this ILICI mouse model[108]. Whether these monocytes were reacting to the established injury or participating in mediating liver cell death is yet to be determined. Currently, there are no mechanistic studies exploring the mode of cell death in ICI irAEs and ILICI, although T-cell mediaed apoptosis is a likely contributor[108]. Using ingenuity pathway analysis on transcriptomes of mice treated with CTLA-4 inhibitor plus IDO inhibitor, Affolter and colleagues identified upregulation of both liver apoptosis and necrosis pathways[108]. Further detailed studies are needed to explore the contribution of various cell death subroutines to ILICI.

Table 2 Mechanisms of immune checkpoint inhibitor toxicity.

Innate immunity	Mechanism
CTLs	- Epitope spreading process[82] - Stimulated proliferation of $CD8^+$ T cells[86,87] - Overlap of T-cell antigens between the tumor microenvironment and tissues with irAEs[62] - ICI immunotherapy overcoming immune tolerance, altering $CD8^+$ transcriptional profile of cytokines[86–89]
T helper and Treg	- ICI treatment leading to a reduction of T-regs and subsequent reduction of anti-inflammatory cytokines[92,93] - Expansion of Th1 cells and increase in pro-inflammatory cytokines leading to activation of CTLs, monocytes and macrophages[62,95,96] - Anti CTLA-4 inhibitor binding to CTLA-4 expressed on Tregs causing antibody-dependent cell mediated cytotoxicity and subsequent Treg depletion[62]
B cells	- Early decline in total circulating B cells, an elevation of $CD21^{lo}$ subtype has been shown following dual checkpoint inhibition therapy[99]. This increase has also been reported in CTLA-4 deficient patients[100]
Cytokines	- Circulating cytokines have been studied as biomarkers and predictors of irAEs[102] - TNF is a potential contributor to irAEs as anti TNF therapy can also prevent prolong steroid use and treat certain gastrointestinal irAEs such as enterocolitis[106]. Anti-TNFs are not recommended for hepatitis due to concerns with hepatotoxicity[107]

CTLA-4, cytotoxic T lymphocyte antigen; CTLs, cytotoxic T lymphocytes; ICI, immune checkpoint inhibitor; irAEs, immune related adverse events; TNF, tumor necrosis factor.

Gudd and colleagues evaluated the peripheral blood monocytes (PBMCs) of 22 patients with ILICI with the majority undergoing dual immunotherapy regimen[96] compared to 7 patients who received ICIs but did not develop hepatotoxicity and 19 healthy donors. Total peripheral monocytes demonstrated a marked reduction, although the classic monocyte subtype ($CD14^{high}$ $CD16^{low}$) was elevated[96]. Patients with ILICI were reported to have higher soluble and cell surface expression of CD163, a marker for monocyte and macrophage activation[96]. The functional and phagocytic capacity of these monocytes for microbial removal was maintained[96]. Gene expression profiling in monocytes demonstrated downregulation of immune modulators such as G-protein coupled receptor 183 (GPR183), an IFNγ and TLR response modulator, and prostaglandin endoperoxide synthase 2 (PTGS2), an enzyme involved in prostaglandin synthesis in monocytes[96]. Interestingly, the CD163 level, as well as the number of classical monocytes was positively correlated with the ALT while the non-classical monocytes were negatively correlated with injury and ALT values[96]. *In vitro*, monocyte derived macrophages isolated from these patients displayed $CD163^{high}CCR2^{high}$, indicating an activated phenotype and tissue homing properties. Furthermore, they secreted inflammatory cytokines such as IFN-γ, IL-1β, IL-6, IL-12p70 and TNF following lipopolysaccharide stimulation[96]. In addition to the innate cell changes, Gudd and colleagues noted increased levels of perforin and granzyme B + cytotoxic $CD8^+$ T cells in patients with ILICI compared to those who received checkpoint inhibitors with no liver toxicity, and healthy controls[96,109]. Using immunohistochemistry the authors showed that the $CD68^+/CCR2^+/CD163^+$ macrophages and the $CD8^+$ T cells were enriched in livers of ILICI patients and they colocalized in areas of injury, suggesting cross talk between the innate and adaptive immune system in patients with hepatotoxicity from ICIs[96]. This study highlights the possible contribution of innate immunity in the pathogenesis of ILICI.

Liver biopsies of patients with ILICI may hint towards a potential immune mechanism. There is no uniform presentation of liver injury on biopsy and histologic findings of these patients are heterogeneous. Such heterogeneity may be due to the enigmatic pathogenesis of ICI-induced hepatotoxicity or due to the limited number of patients studied thus far[110–112]. Liver parenchymal injury caused by anti-PD-1 has primarily been described as lobular hepatitis, with mild lobular and periportal infiltration and patchy foci of necrosis[113]. It may co-occur with bile duct injury, and the lymphocytic cholangitis may lead to vanishing bile duct syndrome[114]. The histopathologic features in three reported cases by Doherty et al.[114] revealed diverse degrees of bile duct damage and ductopenia, including one consistent with vanishing bile duct syndrome. Steatosis and fibrin ring granulomas have not been commonly reported in hepatitis induced from ipilimumab

monotherapies. However, both monotherapies and combination therapies have been characterized by pan lobular necrosis with a predominantly lymphocytic cell infiltrate and a paucity of plasma cells[113]. Whether the observed lobular necrosis is the primary mode of hepatocyte cell death (classic MPT-mediated necrosis) or whether this observed pathology is indeed secondary necrosis which occurs following apoptosis is yet to be studied[115].

One recent clinical study by De Martin et al.[116] analyzed the histological features of acute hepatitis that developed on average within five weeks following immune therapy. Of these patients, those treated with anti-CTLA-4 monotherapy or in combination with anti-PD-1 therapy demonstrated fibrin ring granulomas and central vein endotheliitis[116]. Histological analysis of hepatic tissue following anti-PD-1 therapy revealed lobular hepatitis consistent with prior studies[113]. Immunostaining demonstrated a significant distinction between the types of infiltrated lymphocytes. In anti-CTLA-4 therapy induced hepatotoxicity infiltrating cells primarily consisted of $CD8^+$ CTLs; however, liver biopsy findings of patients with ILICI from anti-PD-1/PD-L1 therapy revealed that $CD8^+$ and $CD4^+$ T lymphocytes were equally present in liver biopsies. Importantly, while ILICI shares some features of autoimmune hepatitis (AIH), the number of plasma cell infiltrates in this study was far less than what is seen in classic AIH[116]. In animal models of ICIs both $CD4^+$ and $CD8^+$ T cells infiltrates have been noted in the liver[117]. Johncilla et al.[118] observed hepatocyte regenerative changes, including increased cell size, binucleation, prominent nucleoli, mitoses, and focal apoptosis. Ballooning degeneration and multifocal hepatocyte apoptosis were present in all eleven cases who developed ILICI with anti-CTLA-4 therapy (ipilimumab). Mild to moderate endothelialitis of central veins with perivenular destruction was present in most cases, and mild bile ductular inflammatory reaction was also noted[118]. $CD8^+$ T cells were detected predominantly in 4 out of 6 cases of pan-lobular hepatitis, with few $CD4^+$ T cells detected, and one patient showed a mixed population of $CD4^+$ and $CD8^+$ T cells. Scattered $CD20^+$ B cells were also detected in all cases, and similar to the observation by Gudd and colleagues intrasinusoidal $CD163^+$ macrophages were detected in half of the cases[118]. Further studies on patients with ICI hepatitis are necessary to determine the role of B cells and the importance of innate immune cells in this form of toxicity.

Interestingly, in mice, it has been shown that immune infiltration following ICI therapy may result in heterogeneous organ-based outcomes based on the strain and genetic background. For instance, PD-1 injection with Complete Freund's Adjuvant boosters in the C57BL/6 mouse strain causes liver and lung immune infiltration similarly to the MRL/MpJ strain, which can also cause infiltration in the pancreas[117]. On the other hand, BALB/c mice manifest no immune infiltration, and SWR/J mice show infiltration only in the colon. A standard animal model is yet to be identified to investigate this type of injury[117]. The predominant infiltrates in the liver, lung and pancreas were $CD4^+$ and $CD8^+$ T cells. Additionally a significant number of $CD19^+$ B cells and F4/80 macrophages were also reported to be present in the liver and colon of mice treated with dual PD-1 and CTLA-4 antibodies[117]

4.1.5. Modulation of immune tolerance by ICIs, predisposes to idiosyncratic DILI

Checkpoint inhibitor blockade has been used to prevent adaptation and block immunotolerance as well as to illicit an immune reaction to known hepatotoxic drugs. For many years, studying idiosyncratic DILI (IDILI) has been hindered by the inability to recapitulate this form of hepatotoxicity in animal models. IDILI is by definition dose-independent (above a certain threshold), unpredictable, and with a variable and often long latency[48]. The rare occurrence of hepatotoxicity is in large part due to the liver's ability to dampen immune responses *via* induction of tolerance, a phenomenon often referred to as adaptation. There are many instances of this clinically, with adaptation to isoniazid being the classic example[119]. Since the immune checkpoints function to induce tolerance, it has been hypothesized that the break of this tolerance could promote an aberrant immune reaction against a foreign antigen such as a drug or its metabolite, which can be termed "defective tolerance". Further supporting a possible role for immune checkpoint defects contributing to the mechanism of IDILI is the inflammatory stress hypothesis, which postulates that inflammation during drug therapy could interact with the action of the drug and unleash an inadvertent immune response, thereby predisposing to IDILI[120–122]. Inflammation can lead to altered cellular signaling, as well as an accumulation of cytokines and immune cells in the microenvironment[121]. Inflammation can also alter the expression of drug-metabolizing enzymes and drug transporters and repress the liver's repair capability[121]. Since ICIs act by activating T cell responses, promoting CTL activation and inhibiting Tregs, it is feasible that they may also cause inflammatory stress leading to aberrant IDILI and hepatotoxic reactions to otherwise innocuous medications.

An example of this type of liver injury was recently demonstrated using amodiaquine (AQ)[123]. AQ administration to C57BL/6 mice results in mild ALT abnormalities that resolve with time, suggesting adaptation. To test whether a break of immune tolerance is implicated in AQ IDILI, Metushi et al.[123] treated $PD1^{-/-}$ mice with CTLA-4 inhibitor prior to introducing the drug AQ. Indeed, blocking the immune checkpoints resulted in hepatitis, immune cell infiltration in the liver and elevated ALT, which did not resolve with the discontinuation of AQ. PD-$1^{-/-}$ mice treated with AQ and anti-CTLA-4 stained positive for Ki-67, CD45R, Mac2, CD4, and CD8[109]. The number of Tregs and the levels of perforin and granzyme B secreted by $CD8^+$ T-cells were also elevated in these mice. The inflammatory infiltrate consisted of $CD8^+$ T cells and macrophages and correlated with areas of necrosis[109]. As previously discussed, APCs in the liver including KCs and DCs express PD-1/PD-L1, inhibit the activation of $CD8^+$ T cells, and promote Treg function. By inhibiting both checkpoints in this model, Tregs were suppressed and effector $CD8^+$ T cells were activated against AQ, leading to hepatocyte injury[109]. The same group further investigated the mechanism of liver injury by extending the duration of AQ treatment to 10 weeks. The mice did not present with liver failure, although bilirubin levels were increased[124]. The percentage of hepatic CD4, CD8, Th17, and Treg cells was elevated, and NK cells were significantly decreased[124]. To examine the role of $CD8^+$ T cells, a $CD8^+$ T cell antibody was used to deplete this population[124]. Interestingly, depletion of CTLs abrogated liver injury, suggesting a pivotal role for $CD8^+$ T cells in mediating hepatotoxicity to AQ[124]. The authors went on to test the contribution of innate immunity to liver injury in this model and noted that $CCR2^{-/-}$ mice (which have reduced monocyte recruitment to sites of injury) treated with AQ developed mild liver injury which resolves with continuation of drug therapy similar to WT animals[125]. The $CCR2^{-/-}$ animals had less infiltrating NK cells but this was not statistically significant[125]. In PD-$1^{-/-}$ mice treated with CTLA4 inhibitor, the administration of anti-CTLA-4 significantly decreased AQ

induced liver injury, as well as infiltration macrophages, suggesting a possible role for innate immunity[125].

Blocking CTLA4 in PD1$^{-/-}$ mice also caused liver injury from nevirapine and isoniazid (INH) and was associated with increased NK cells (in the case of INH) and increased CD8^{+} CTLs (with nevirapine)[68]. The same was true for epigallocatechin gallate (EGCG), the major catechin in green tea[126]. While WT mice displayed no liver injury from EGCG, female PD-1$^{-/-}$ mice treated with CTLA-4 inhibitor displayed foci of inflammation and modest elevations in ALT[126].

While these studies were carried out as models of IDILI, the fact that multiple drugs illicited an immune reaction in the liver of mice treated with immunotherapy suggests that it is possible that ILICI occurs in certain individuals due to aberrant targeting of adaptive and innate immune cells to stimuli such as drugs and xenobiotics. And therefore, an additional "hit" such as the formation of drug metabolites or neoantigens that would have otherwise gone unnoticed by the immune system may be necessary to result in immunotherapy induced hepatotoxicity and ILICI. This may explain why some patients develop ILICI while others do not. Further studies are necessary to study this hypothesis.

5. Summary and conclusions

Immune checkpoints are negative regulators of T cell responses. They are essential for the maintenance of immune tolerance and to prevent autoimmunity. Cancer cells develop ways of evading clearance by CTLs, by upregulating these immune checkpoint receptors. Blocking the immune checkpoints and harnessing the cytotoxic properties of CD8^{+} T cells to eliminate cancer cells has proven effective and has revolutionized cancer immunotherapy. However, blocking immune tolerance comes with the cost of inducing autoimmune reactions that target various organs, including the liver[1]. As severe grade 3 to 4 ILICI from immunotherapy requires discontinuation of this potential lifesaving treatment, understanding the underlying mechanism of ILICI and developing therapies to prevent and treat hepatotoxicity without decreasing the effectiveness of antitumor treatment is of critical necessity. Liver related toxicities are increasingly recognized and with the approval of ICIs for HCC[79] and in patients with underlying liver disease understanding this form of immune mediated hepatotoxicity is of paramount importance. A recent meta-analysis of checkpoint inhibitor studies in HCC demonstrated that the risk of liver injury as measured by liver enzyme elevations (AST and ALT) in patients with HCC is three times higher than other solid tumors[127]. Certain characteristics such as dual ICI therapy, male sex, younger age have been associated with increased risk of hepatotoxicity, however, the reason why certain individuals develop ILICI while others do not, is not clear.

Using animal models and patient samples, it is known that ILICI is at least in large part the result of CTL activation. CD8^{+} T lymphocyte infiltrates have been reported in the livers of humans and mice treated with ICIs and who present with elevated transaminases[96,108,128]. Involvement of CD4^{+} T helper cells and Tregs in both preclinical models and clinical settings has been noted[62,117]. CTLA-4 antibodies are known to bind Tregs mediating their depletion and altering immune homeostasis[62]. ICIs also affect the composition of the T helper population including activation of Th1 and Th17 cells which go on to secrete proinflammatory cytokines such as IL-12, IL-17, IFN-γ and TNF. The impact of PD-1 blockade on the function of Th1 cells remains to be elucidated; however, PD1/PD-L1 has been shown to promote Th17 differentiation to Tregs[62]. Patients with irAEs during immune checkpoint treatment develop a significant increase in Th17 cell population and display elevated levels of baseline IL-17[95,102,129]. These proinflammatory cytokines can subsequently activate an innate immune response by recruiting NK cells and macrophages contributing to liver injury[62]. Recently, a role for monocyte derived macrophages and soluble CD163 levels has been suggested in ILICI[96]. Liver biopsies of these patients revealed the presence of both CD8^{+} CTLs and CD68^{+} macrophages in proximity, suggesting that immune cells work in concert and the adaptive innate cross talk is important in the pathogenesis of ILICI. Whether ICIs cause CTLs to directly target hepatocytes through FAS/FASL or perforin/granzyme B or hepatocytes are targeted due to ICI effects on Tregs and T helper cells resulting in immune dysregulation and the activation of innate immunity has not been fully elucidated. It is likely that multiple pathways are at play and that immunotherapy induces changes to the inflammatory environment broadly activating simultaneous pathways. Therefore, it is reasonable to hypothesize that innate and adaptive immunity cross talk leads to hepatocyte injury and cell death.

There are still many unknowns in terms of the molecular mechanisms of ILICI. The nature of the immune response is not fully understood, and it is not clear if just blocking the immune checkpoint is sufficient to drive significant liver inflammation and autoimmunity, or a secondary "hit" such as a drug or environmental trigger or xenobiotic is necessary for hepatoxicity. Furthermore, what happens after the immune system is activated in the liver microenvironment and whether the toxicity is directed at hepatocytes alone or liver non-parenchymal cells, which abundantly express PD-L1, is not clear. The signaling events downstream of CTL and macrophage activation, and which cytokines are key in their crosstalk, requires further study. As more patients are treated with immunotherapies and our experience with them grows in patients with HCC and chronic liver disease, deeper understanding of underlying mechanisms is vital so that tailored therapies to mitigate immune toxicities can be developed.

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Author contributions

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Conflicts of interest

The authors declare no conflicts of interest.

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